Published in final edited form as:

J Appl Physiol. 2008 May ; 104(5): 1485–1494.

Spatial and temporal heterogeneity of ventilator-associated lung injury after surfactant depletion

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Abstract

Volutrauma and atelectrauma have been proposed as mechanisms of ventilator-associated lung injury, but few studies have compared their relative importance in mediating lung injury. The objective of our study was to compare the injury produced by stretch (volutrauma) vs. cyclical recruitment (atelectrauma) after surfactant depletion. In saline-lavaged rabbits, we used high tidal volume, low respiratory rate, and low positive end-expiratory pressure to produce stretch injury in nondependent lung regions and cyclical recruitment in dependent lung regions. Tidal changes in shunt fraction were assessed by measuring arterial P_{O_2} oscillations. After ventilating for times ranging from 0 to 6 h, lungs were excised, sectioned gravitationally, and assessed for regional injury by evaluation of edema formation, chemokine expression, upregulation of inflammatory enzyme activity, and alveolar neutrophil accumulation. Edema formation, lung tissue interleukin-8 expression, and alveolar neutrophil accumulation progressed more rapidly in dependent lung regions, whereas macrophage chemotactic protein-1 expression progressed more rapidly in nondependent lung regions. Temporal and regional heterogeneity of lung injury were substantial. In this surfactant depletion model of acute lung injury, cyclical recruitment produced more injury than stretch.

Keywords

acute lung injury; chemokines; inflammation

Excessive stretch of pulmonary tissues has been proposed as a mechanism to explain the progression of lung injury during mechanical ventilation [ventilator-associated lung injury (VALI)] (11,12,29,38). In addition to experimental evidence demonstrating injurious and

proinflammatory effects of stretch from large tidal volume ventilation (2,8,12,13,18,24,32, 39,47,50,52), a recent landmark clinical study demonstrated improved outcome with a ventilatory strategy that focused on reduced tidal volumes (1). A second proposed mechanism of VALI is atelectrauma associated with repetitive recruitment (opening) and derecruitment (collapse) of alveoli during tidal breathing (a phenomenon sometimes referred to as cyclical recruitment) (11,29). A major shortcoming in defining the contribution of atelectrauma has been the challenge of quantitatively and continuously assessing cyclical recruitment during tidal ventilation. Evidence supporting a role for cyclical recruitment in VALI has relied predominantly on experimental studies that showed a beneficial effect of positive end-expiratory pressure (PEEP) (4,8,12,18,25,33,39,46,47,52). This effect of PEEP has been attributed to reduction of atelectrauma, although these experiments did not directly measure cyclical recruitment.

Benefits of PEEP in clinical studies have been more elusive. Prior studies have suggested that high PEEP can be a beneficial component of protective ventilation strategies when adjusted according to individual lung mechanics (3,51), an effect attributed to reduced cyclical recruitment. One recent randomized controlled trial, however, suggested little or no survival benefit to high vs. low PEEP (7).

Although both stretch and cyclical recruitment are implicated in VALI, the relative contribution of each mechanism is currently unknown. Defining the impact of stretch vs. atelectrauma has important clinical implications. During mechanical ventilation, strategies to minimize stretch may exacerbate cyclical recruitment, and, conversely, minimizing cyclical recruitment through the use of PEEP may increase stretch (17,19,29). Without a clear understanding of the contribution of stretch vs. atelectrauma, optimization of ventilator strategies to maintain recruitment and prevent VALI will remain a challenge.

In a saline lavage, acute lung injury model, we investigated the relative contribution of volutrauma vs. atelectrauma to regional inflammation. We intentionally adjusted the ventilator to produce both stretch and cyclical recruitment and used intra-arterial Po_2 (Pa_{O_2}) oscillations to confirm and monitor cyclical recruitment (5). We hypothesized that, in serial gravitational lung sections, the tissue chemokine and neutrophil inflammatory responses would be different in regions of the lung exposed to volutrauma vs. atelectrauma.

METHODS

Animal preparation

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Twenty-three female, specific pathogen-free, New Zealand White rabbits (average weight 3.7 ± 0.4 kg) were entered into the study. Twenty of the rabbits were randomly assigned to be ventilated for 0, 1.5, 3, or 6 h following lavage, and three rabbits were assigned to the control group. Each rabbit was sedated with 140 mg of ketamine intramuscularly (im) and 10 mg of xylazine im, then placed in dorsal recumbence for the duration of the study. After insertion of an intravenous (iv) catheter into an ear vein, general anesthesia was induced with an infusion of thiopental (starting at $5\text{--}10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) and a bolus of iv fentanyl ($25\text{--}50 \mu\text{g}$). General anesthesia was maintained with a titrated infusion of thiopental iv and titrated boluses of iv fentanyl. A pulse oximetry probe (N-100, Nellcor, Pleasanton, CA,) was applied to the left forepaw. A heating pad was used to maintain rectal temperature at $38.0 \pm 0.9^\circ\text{C}$. After midline tracheostomy, a 4.0-mm inner diameter endotracheal tube was sealed in the trachea with umbilical tape. During the rest of the preparation and experiment, all animals were ventilated in a pressure-controlled mode with an inspired O_2 fraction of 1.0 (Servo 900C, Siemens, Germany). The three control rabbits were killed immediately after placing a peripheral (ear) arterial catheter. For the remaining 20 rabbits, a catheter for arterial pressure

monitoring was inserted in the femoral artery by surgical cutdown, and a catheter was placed into the saphenous vein for drug and fluid administration. A 6-Fr introducer (Edwards Lifesciences) was placed in the right jugular vein after exposure by cutdown to allow for a 5-Fr, balloon-tipped pulmonary artery catheter (Edwards Lifesciences) to be advanced into the right ventricle as guided by the pressure waveform. A 20-gauge catheter for insertion of the Pa_{O_2} probe was placed by cutdown into the right carotid artery. After completion of all surgical procedures and establishment of the appropriate thiopental infusion rate and fentanyl dosing intervals for deep general anesthesia, the animals were paralyzed with 0.76 mg/kg of pancuronium iv. Neuromuscular blockade was maintained at an overall average administration rate of $0.38 \pm 0.22 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$.

Fluid management

The fluid management protocol was similar to our previous study (5). Maintenance infusion of intravenous crystalloids (Normasol R solution, Abbott Laboratories, North Chicago, IL) was provided at 25–30 ml/h. Throughout the study, 10- to 20-ml boluses of colloid (6% Hetastarch, Abbott Laboratories) were administered when there were sustained respiratory variations of the blood pressure waveform or persistent decreases in systolic blood pressure to $<70 \text{ mmHg}$. Epinephrine infusion was titrated for persistent hypotension following three boluses of colloid within a 15-min period.

Pa_{O_2} probe

The intra-arterial oxygen probe used for these studies was a fiber-optic, fluorescence-quenching probe with an uncoated ruthenium complex at the probe tip (FOXY-AL300, Ocean Optics, Dunedin, FL) as previously described (5). The fiber-optic probe was connected to an SF2000 spectrometer, with computer interfacing via an ADC1000-USB analog-to-digital system (Ocean Optics). Commercially available software (OOISensors 1.0, Ocean Optics) was used in real-time mode to continuously display Pa_{O_2} measurements at a digital sample rate of 3.4 samples/s.

The probe was advanced into the brachiocephalic artery of the rabbits through the 20-gauge catheter placed in the right carotid artery. Similar to the previously described method (5), two criteria were used to confirm proper positioning in the lumen of the brachiocephalic artery: 1) constant amplitude of the respiratory oscillation in Pa_{O_2} as the probe was advanced and withdrawn over at least 0.8 cm and 2) adequate time response in the mean Pa_{O_2} signal to a step change in inspired O_2 fraction.

Calibration of the Pa_{O_2} probe was carried out at the beginning of each experiment, before lavage. In the normal lung, the ventilator is easily adjusted to provide nearly complete lung recruitment, minimal cyclical recruitment, and therefore minimal Pa_{O_2} oscillations. The steady signal from the Pa_{O_2} probe was recorded during a period of 100% oxygen breathing and a period of air breathing. For the two-point calibration, the recorded signal intensity and the corresponding Pa_{O_2} from conventional blood gas analysis were used to derive parameters in the Stern-Volmer equation that made the Pa_{O_2} calculated from the probe signal intensity match the Pa_{O_2} from arterial blood gas analysis. The Stern-Volmer equation relates signal intensity I to Po_2 : $I_0/I = 1 + k\text{Po}_2$, where I_0 is the intensity at zero Po_2 and k is a calibration constant.

Lung lavage

With the rabbits in dorsal recumbence, lung lavage was achieved by steady injection (over $\sim 45 \text{ s}$) of 100 ml of warmed balanced crystalloid solution (Normosol-R) into the endotracheal tube. The lavage fluid was immediately drained by gravity. The Pa_{O_2} and blood pressures were continuously monitored during the procedure. After drainage of each lavage, a recruitment maneuver (inspiratory hold for 10–15 s, with confirmation of recruitment from the Pa_{O_2} probe)

was performed. As soon as the blood pressure and Pa_{O_2} recovered, the lavage procedure was repeated for a total of three lavages over a total period of ~10–20 min.

Ventilator management

After lavage, the ventilator was adjusted to reach, as closely as possible, targeted goals of 10% fixed shunt fraction, and shunt fraction oscillating between 30% at end-expiration and 10% at end-inspiration. Shunt fraction was assessed continuously from the high time resolution Pa_{O_2} measurements and the intermittent mixed venous blood gas data, using the algorithms of Olszowka and Farhi (36) to relate O_2 and CO_2 tensions to content. Pa_{O_2} , respiratory mechanics, and hemodynamic data were monitored continuously and recorded every 30 min. Ventilator parameters were adjusted every 15 min within the allowable ranges of maximum plateau pressure of 35 cmH_2O , minimum PEEP of 2 cmH_2O , and minimum respiratory rate of 5 breaths/min.

Respiratory, hemodynamic, and blood gas monitoring

Respiratory mechanical data were monitored continuously from the ventilator display and manually recorded every 30 min. Hemodynamic data were monitored continuously and recorded every 30 min. Arterial and mixed venous blood gases were drawn for probe calibration, immediately after lavage, hourly after lavage (except the 6-h group, where they were obtained every 2 h), and at the end of the experiment. Samples were rapidly drawn into heparinized syringes at the end-expiratory nadir in Pa_{O_2} (37) and analyzed immediately on a blood gas analyzer (Stat 9, NOVA Biomedical, Waltham, MA). Blood samples were drawn in EDTA for complete blood counts following catheter placement, following lavage and at experiment end. Counts were performed on an automated complete blood count analyzer that has been validated for domestic rabbits (Cell Dyne 3500, Abbott Diagnostics, Santa Clara, CA).

Lung isolation and processing

At the end of the experiment, the rabbits were heparinized and exsanguinated through the femoral arterial line, while volume was replaced with colloid via a peripheral venous catheter, maintaining right ventricular pressure at or below the prehemorrhage baseline. Volume replacement during blood withdrawal allowed for effective hemodilution and removal of blood elements from the pulmonary vasculature. The trachea was clamped at 30 cmH_2O of continuous positive airway pressure, and the chest was opened and the lungs and heart were removed en bloc. The lungs were cleaned of excessive tissue and rinsed with Normasol R. The right mainstem bronchus was double ligated, and the lung was removed and immersed in isopentane and dry ice for rapid freezing (40). After 3–5 min, the lung was removed from the bath and sectioned into eight to nine horizontal sections from ventral (nondependent) to dorsal (dependent). Equal section widths of 5.5 mm were approximated using an egg slicer; tissue was cut with a high-profile disposable microtome blade (Surgipath Medical, Richmond, IL). Immediately after the sections were cut, they were placed on preweighed plastic weighing vessels and immersed in liquid nitrogen. All sections were then stored at -70°C until further processing.

The left lung was removed and insufflated with a 10% formalin solution at 30 cmH_2O for 30 min and then immersed in a bath of 10% formalin for a minimum of 2 wk.

Processing of the right lung

Right lung samples were processed using an adaptation of previously reported methods (23). Frozen slices from the right lung were kept frozen between processing steps on dry ice. After weighing, individual slices were pulverized inside a 4°C cold room using a manual mechanical

crushing device (BioPulverizer, BioSpec Products, Bartlesville, OK), which had been prechilled in liquid nitrogen. The powdered tissue was transferred into preweighed cryogenic containers (Nalge Nunc, Rochester, NY) for samples ≤ 3.0 g or 50-ml centrifuge tubes for samples > 3.0 g. The pulverized tissue was weighed, and the weight was recorded. Samples were returned to -70°C until additional processing. Samples were reconstituted with 4 ml of protease inhibitor buffer [100 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 mM EDTA, 10 μM trans-epoxy-succinyl-L-leucylamido(4guanidino)butane in water] for each gram of tissue. After vortexing, the samples were subjected to three freeze-thaw cycles: frozen at -70°C and thawed on ice and sonicated on ice for 15 s at 6 W (60 Sonic Dismembrator, Fisher Scientific). After the final thaw, samples were homogenized on ice using a continuous duty DC motor (Glas-Col, Terre Haute, IN). Samples (minimum of 300 μl) were removed for MPO (myeloperoxidase) assay. The remaining homogenate was centrifuged at 28,770 g (16,000 revolutions/min) for 10 min at 4°C (Sorvall Super T21). After the final ultracentrifugation of the homogenized sample, the pellet and supernatant were separated, and the pellet was weighed. The volume of supernatant was measured, aliquotted, and stored at -70°C .

Pellet weight and the weight of the starting sample were used to calculate the original weight of solids in the starting sample and a wet-to-dry equivalent ratio for assessing edema (see supplemental data available at the *Journal of Applied Physiology* website for details).

Chemokines and cytokine ELISA

Supernatant samples for ELISA assay of rabbit monocyte chemoattractant protein-1 (MCP-1), growth-related oncogene (GRO), IL-8, and TNF were stored at -70°C until being shipped on dry ice to the Pulmonary Research Laboratories, Seattle Veterans Affairs Medical Center, Seattle, WA. Assays were performed following standard methods as previously described (21,22). Samples for MCP-1 and GRO were assayed at 1:2 and 1:20 dilutions in buffer. Samples for IL-8 were assayed at 1:20 and 1:200 dilutions. TNF was assayed at a 1:4 dilution. The measured chemokine concentrations in supernatant were used to calculate the starting mass of chemokines in the original sample, which was normalized to the weight of solids in the original sample (see supplemental data available at the *Journal of Applied Physiology* website for details).

Inducible NO synthase activity

The assay of inducible NO synthase (iNOS) activity was adapted from Weinberg et al. (53). Briefly, 30 μl of the lung lysis solution was removed and incubated with 20 μl of reaction buffer [final concentration 50 mM HEPES (pH 7.4), 200 μM NADPH (Sigma), 1 mM dithiothreitol (DTT, Fisher Scientific, Pittsburgh, PA), 10 μM FAD (ICN), 100 μM tetrahydrobiopterin (BH4, Sigma), 10 μM L-arginine (Sigma), 0.4 μl of [^{14}C]arginine labeled in the guanido position at a radioactive concentration of 50 $\mu\text{Ci/ml}$ and specific activity of 396 $\mu\text{Ci}/\mu\text{mol}$ (Amersham, Arlington Heights, IL)]. Samples were incubated in duplicate for 30 min at room temperature and then loaded on a 0.4-ml DOWEX AG cation exchange resin (50W-X8, Bio-Rad) column and washed two times with 3 ml of distilled H_2O . Measurement of L-citrulline was determined by lack of adherence to the DOWEX resin. Specific activity was measured following the addition of 16 ml of Luminex (ICN) scintillation fluid on a Beckman LS6500 scintillation counter. Counts per minute were normalized to sample solids for each sample.

MPO assay

Homogenate samples were resuspended 1:1 (vol:vol) in HTAB (50 mM potassium phosphate, dibasic, pH 6.0; 0.5% hexadecyltrimethyl ammonium bromide, 5 mM EDTA buffer) buffer for peroxidase activity by the Amplex red hydrogen peroxide/peroxidase kit (A22188, Molecular Probes). Suspensions were clarified by centrifugation at 14,000 revolutions/min for 10 min at 4°C . Supernatant (50 μl) was added to 50 μl of 100 μM Amplex red with 2 mM

H₂O₂ for analysis. A standard curve was generated from serial dilutions of 200 μ M resorufin. Duplicate samples in a 96-well plate format were incubated for 30 min in the dark, and then they were read on a fluorescence plate reader (Molecular Devices SpectraMax Gemini XS) at 450 nm. The optical density and activity were captured with Software Pro software. Results were normalized to the amount of total protein in the supernatant, which was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). A protein standard curve was generated using bovine serum albumin.

Histopathology for the left lung

A piece of fixed tissue, 15 mm wide $\times$ 20 mm deep $\times$ the full height of the lung block, of the formalin fixed left caudal lobe was removed using the hilus as the central landmark. This section was then further sectioned into eight to nine serial slices from ventral (nondependent) to dorsal (dependent). Tissue slices were embedded with the dorsal surface of the slice down. Four- to 6- μ m tissue sections were taken from the dorsal surface of each section, and stained with hematoxylin and eosin. The stained slides were randomly assigned numbers to allow for blinded review. For each slide, 10 random, nonoverlapping, 150 $\times$ 200- μ m images were captured by digital microscopy. The images were saved and scored using image analysis software (ImagePro Plus software by Media Cybernetics, Silver Spring, MD). Manual enumeration of alveolar neutrophils, degree of interstitial edema (no septal thickening = 1, patchy = 2, diffuse = 3), presence of red blood cells, and subjective assessment of aeration (fully aerated = 1, partially collapsed = 2, complete collapse = 3) was performed by one of two individuals. The scoring system was validated by review of selected sections with a veterinary pathologist. The average of the 10 slides is reported for each section.

Statistical analysis

All descriptive data are reported as mean $\pm$ SD, unless the data failed tests for normality, in which case the median and interquartile range are reported.

All regression testing was carried out by stepwise forward regression with time and fractional height in the starting model. Candidates for entry were an interaction term (time $\times$ fractional height), a second-order term for time (time²), and experimental order number. The *F* value to enter the regression was set at 4.0, whereas the *F* value to remove the variable from the regression was 3.9. Order number was not a significant predictor for any data set, except for alveolar neutrophil count.

Data were transformed as needed to satisfy normality testing of residuals and equal variance testing. Residuals were also inspected visually for each data set. The data from GRO and the wet-to-dry equivalents (W/De) did not require transformation. The square root transforms ($X^{0.5}$) were used for IL8, iNOS, and intra-alveolar neutrophil count. MCP-1 required $X^{0.25}$. For MPO, no suitable transform could be found; MPO data were analyzed by nonparametric ANOVA (Kruskal-Wallis) for time, and nonparametric *t*-test (Wilcoxon signed rank sum) for top half vs. bottom half of lung. Peripheral neutrophil counts over time and across groups were analyzed by two-way ANOVA and pairwise multiple comparisons by the Holm-Sidak method.

Data for all lung slices were averaged together for each control and *time 0* lavage animal. Control values were compared with *time 0* lavage animals by parametric *t*-test. All data sets satisfied normality and equal variance without transformation, except the MPO data, which required a square root transform. All statistical analyses were performed with SigmaStat 3.11, Systat Software, San Jose, CA.

RESULTS

Three rabbits assigned to the zero injurious ventilation time (*time 0*), four rabbits in the 1.5-h ventilation group, three rabbits in the 3-h ventilation group, and two rabbits in the 6-h ventilation group completed the protocol. All values are reported as mean $\pm$ SD unless otherwise noted. The total lavage volume of 81.4 ± 8.7 ml/kg, divided into three sequential lavages, produced a mild to moderate lung injury, with an average Pa_{O₂}-to-inspired O₂ fraction ratio postlavage of 203 ± 92 at a median PEEP of 2 cmH₂O (range 2–4 cmH₂O). The low respiratory rate (median 8 breaths/min; interquartile range 6.6–8.5), low PEEP (median 3 cmH₂O, interquartile range 2–3), and high tidal volume (mean 28 ± 5 ml/kg) ventilator settings used to obtain the target fixed and oscillating shunt fractions are detailed in supplemental data (available online at the *Journal of Applied Physiology* website). On average, across groups and time, the fixed shunt fraction was $9.4 \pm 2.7\%$, and the amplitude of the oscillating shunt fraction was $19.3 \pm 9.5\%$.

The spatial and temporal expression patterns for the chemokines MCP-1, IL-8, and GRO are graphically displayed in Fig. 1. All chemokines demonstrated a time-dependent expression; both IL-8 and MCP-1 expression varied significantly between dependent and nondependent regions (Table 1), with greater IL-8 expression in the dependent regions and greater MCP-1 expression in the nondependent regions. TNF was not detectable in any sample at any time point (data not shown). Regional enzyme activity for MPO and iNOS are presented in Fig. 2. The signal-to-noise ratio in both of these assays was low, but there was a significant influence of time on iNOS activity (Table 1). The W/De for the serial slices are presented in Fig. 3. The dependent regions had significantly higher W/De ratios, and the dependent vs. nondependent slice location had significant interactions with time (Table 1). Qualitative edema scores trended in the same direction as W/De, but no changes achieved statistical significance. Eight to nine histopathological sections were obtained from the left caudal lung lobe for all rabbits. Plots of the regional intra-alveolar neutrophil counts, averaged from 10 microscopy fields per section, are presented in Fig. 4. The accumulation of intra-alveolar neutrophils was significantly influenced by time and region of the lung. Figure 5 shows representative micrographs of nondependent and dependent regions.

Table 1 presents a summary of statistical analysis by stepwise forward regression for regional expression of chemokines (MCP-1, IL-8, and GRO), enzyme activity (iNOS), and markers of lung injury (W/De and alveolar neutrophils), where the effects of time and fraction of total lung height for each slice (with 0 as most nondependent and 1.0 as most dependent) and their interactions were tested. A second-order term for time (time²) was also included to test for nonlinearity in time dependence.

To assess the effects of lavage, results from controls vs. *time 0* were compared. For all data (chemokines, enzyme activity, W/De, and alveolar neutrophils), the differences between *time 0* and control were very small compared with the changes after injurious ventilation. W/De for *time 0* was significantly different than control ($P = 0.004$), and all other comparisons showed no significant differences, although the power to detect differences between *time 0* and control was generally low.

DISCUSSION

Our experiments examined the effect of time and the effect of type of injurious ventilation on progression of lung injury after saline lavage. We adjusted the ventilator to produce cyclical recruitment in dependent lung regions and stretch in nondependent lung regions. Continuous cyclical recruitment throughout the studies was produced by use of large tidal volumes, low PEEP, and low rates, and was documented by monitoring tidal variations in shunt fraction with

a fast Po_2 probe (5). Although the tidal variations in shunt fraction do not identify which parts of the lung are undergoing cyclical recruitment, previous studies in multiple species, examining the lungs at end-expiration by computed tomography (CT) after saline lavage, have without exception localized collapse to dependent regions, with little or no collapse in nondependent regions (30,31,35,42,48). Additionally, portions of the lung contributing to shunt fraction have been demonstrated by PET techniques to be primarily distributed to dependent regions after saline lavage (34). Consistent with these prior studies, a pilot experiment using spiral CT at end-expiratory and end-inspiratory breath-holds in our rabbit lavage model confirmed that atelectasis, when present, is predominantly located in dependent lung regions and that aeration is preferentially distributed to nondependent regions (Fig. S3, supplemental data, available online at the *Journal of Applied Physiology* website). Stretch injury was produced by use of large tidal volumes. The tidal volumes administered in our study were larger than the range of volumes that has been shown in prior studies to produce stretch injury in normal rabbit lungs (2,24,43). In our model, the effective tidal volume in the nondependent regions was likely further increased by the presence of atelectasis sufficient to produce $\sim 10\%$ fixed shunt fraction and by the reduction in compliance in the dependent regions from the saline lavage (which depletes surfactant) and ongoing cyclical recruitment (which inactivates surfactant). The nondependent lung regions in our model were therefore exposed predominantly to stretch injury without cyclical recruitment. In this experimental design, the main comparison of interest is dependent lung regions vs. nondependent lung regions in the same lung, thereby allowing each rabbit to serve as its own control. This data pairing accounts for variability between rabbits and individual systemic responses to lavage injury. The data were analyzed by multiple linear regression, which examines the effects of time, position, and their interaction by looking at the whole data set, and does not individually compare small numbers of rabbits in any time group to any other specific group.

Progression of lung injury was assessed by formation of edema (W/De and edema score), by chemokine expression (IL-8, GRO, and MCP-1), by upregulation of inflammatory enzyme activity (iNOS and MPO), and by alveolar neutrophil accumulation. The W/De results suggest that the VALI was progressing more rapidly in the dependent regions. A similar but nonsignificant trend was observed in the edema scores. Neutrophil accumulation also suggests that the injury was more severe in the dependent regions of the lung. The changes in iNOS and MPO activity did not show any significant variations between upper and lower lung regions, although the power to detect regional differences was low compared with the W/De and PMN data. The chemokine results were more heterogeneous, with increased IL-8 expression in the dependent regions, increased MCP-1 expression in the nondependent lung regions, and fairly uniform GRO expression. Overall, these results suggest that cyclical recruitment of atelectasis resulted in more inflammation than moderate to severe stretch injury, with progression over time for up to 6 h after saline lavage.

The substantial spatial and temporal heterogeneity of indexes of inflammation in our surfactant depletion model of lung injury, ventilated with large tidal volumes and low PEEP, has implications for design and interpretation of studies in similar models of lung injury. Measurements that sample from the entire lung, as in whole lung homogenization, or that sample from large parts of the lung, as in bronchoalveolar lavage fluid, could lose some of this regional information in the spatially averaged sample. Additionally, the assessments of lung injury were markedly dependent on the time of ventilation after lavage, an important point when comparing results from different studies.

One of the remarkable findings of our study was that specific types of injurious ventilation produced unique patterns of chemokine responses. In the cyclically recruited and atelectatic, dependent lung regions, IL-8 expression was most pronounced and MCP-1 expression least pronounced, with the opposite pattern in the stretched, nondependent regions. GRO expression

was relatively homogeneous. Cellular deformation and direct mechanotransduction have been proposed as a fundamental mechanism underlying VALI for both atelectrauma and volutrauma (8,11,38). An identical underlying mechanism for injury, however, would imply that the chemokine responses should be similar. The differing patterns of chemokine expression in the dependent and nondependent lung regions suggest that either different underlying mechanisms were responsible for the inflammatory responses or that other factors, other than cellular deformation, were modulating the inflammatory response. A recent study suggested that shear stress and cyclical stretch have differential effects on small GTPase activation in cultured endothelial cells (41), raising the possibility that airway and alveolar epithelial shear stress associated with atelectrauma could lead to different mechanotransduction pathways than epithelial and endothelial stretch associated with volutrauma.

Alternatively, several other differences between the dependent and nondependent lung regions might have played a role in divergent chemokine expression. The dependent regions of the lung were exposed either continuously (fixed atelectasis) or intermittently (cyclical recruitment) to P_{O_2} near mixed venous. Sustained hypoxia and associated activation of the transcription factor, hypoxia inducible factor-1 α , has recently been recognized to initiate inflammatory signaling cascades in the lung (27). More recently, intermittent hypoxia has been hypothesized to directly modulate inflammatory signals, through increased expression of reactive oxygen species (14) and through hypoxia inducible factor-1 α (54). In regard to intermittent hypoxia, the cyclically recruited lung in our experiments could have been exposed to a very large range of oxygen tensions, approaching 1 atm in inspiration to nearly mixed venous P_{O_2} in expiration. In contrast to the hypoxia and/or intermittent hypoxia in dependent lung regions, the nondependent regions of the lung were continuously exposed to hyperoxia. Hyperoxia has also been hypothesized to incite or modulate early lung injury (43) and cellular inflammatory responses, including upregulation of MCP-1 (9).

Comparison to previous studies

We are not aware of any prior studies in saline lavage models of acute lung injury that have compared progression of lung injury in regions of the lung exposed to documented cyclical recruitment of atelectasis vs. regions of the lung subjected to stretch injury. Definitive experimental documentation of cyclical recruitment requires measurements with adequate temporal resolution, which until recently were not available. Current techniques that could be used to dynamically assess cyclical recruitment include dynamic CT (dCT) (10,31,35), electrical impedance tomography (20), subpleural vital microscopy (44), and rapidly responding Pa_{O_2} probes (5). These tools have recently been used to demonstrate that the time constants for recruitment after an increase in airway pressure, and for collapse after a decrease in airway pressure, are not zero but rather are on the same order of magnitude as the time for a typical breath during conventional mechanical ventilation (5,31,35,45). The presence or absence of cyclical recruitment, therefore, cannot be accurately extrapolated from static end-inspiratory and end-expiratory pressures alone, but during tidal breathing it also depends on the dynamics of collapse and recruitment. To our knowledge, none of these new tools for directly assessing cyclical recruitment have been applied previously to compare the impact of cyclical recruitment to the impact of stretch in VALI.

Three recent studies have used dependent and nondependent tissue sampling in the same lung, similar to our use of sequential slices, to compare stretch injury at the top of the lung to atelectasis, and possibly cyclical recruitment, at the bottom of the lung. Tsuchida et al. studied saline-lavaged rats ventilated with high tidal volumes and low PEEP for 90 min (48). They reported no difference in dependent vs. nondependent wet-to-dry (W/D) ratio, similar to our findings for W/De after 1.5 h of injurious ventilation (Fig. 3). They also reported significantly worse injury scores in nondependent vs. dependent regions (48). Expression of mRNA for the

chemokine MIP-2, which binds to the CXCR₂, participates in neutrophil recruitment, and is considered by some authors to be functionally analogous to IL-8 (18,39), was increased in the nondependent regions. There are several possible reasons for our seemingly divergent results. First, based on the Pa_{O₂} data of Tsuchida et al. (48), it is likely that their animals had a relatively large fixed shunt fraction and a resulting small “baby lung” (17). Therefore, it is likely that their similar tidal volume (25 ml/kg in their study vs. 28 ml/kg in our study) translated to a more severe stretch injury in the nondependent regions. Second, a large amount of fixed, or noncycling, atelectasis in dependent lung regions might not undergo the same injury as the relatively large amounts of cyclically recruited lung in our study. Third, in our study, the VALI clearly progressed over time, and the biggest increases in edema, neutrophil counts, and IL-8 expression in the dependent regions occurred at time points later than 1.5 h (Fig. 1). Fourth, these studies were carried out in different species. Finally, several factors, particularly posttranslational modifications, could lead to divergent regional comparisons when comparing mRNA expression and protein expression. A second study that used dependent and nondependent tissue sampling in the same lung to compare stretch vs. atelectasis, and possibly cyclical recruitment, was the investigation of Simon et al. (42). After 5 h of moderate tidal volume, zero PEEP ventilation in saline-lavaged dogs, they assessed gene expression using cross-species genomic microarrays. They found increased expression of multiple genes, including groupings for chemokine activity and inflammatory response, in the dependent lung regions compared with nondependent lung regions. They also reported more severe injury, by qualitative comparison of histopathology, in the dependent regions. In a third study, and in a different canine model, Broccard et al. (6) applied very high tidal volume, low PEEP ventilation, which has been associated with surfactant inactivation (50). After 6 h of ventilation, they reported increased W/D ratios and histopathology injury scores in dependent regions vs. nondependent regions (6).

One recent study compared the injurious effects of cyclical recruitment, directly documented by vital microscopy, with ventilation without cyclical recruitment. Steinberg et al. studied pigs after surfactant deactivation and compared ventilation with low PEEP (3 cmH₂O) to ventilation with high PEEP (15 cmH₂O) (44). Subpleural vital microscopy documented the presence of cyclical recruitment in the low PEEP group and its absence in the high PEEP group. Cyclically recruited lung had significantly increased edema scores on histopathology after 4 h of ventilation, consistent with our finding of increased W/De over time in the dependent lung regions. Although they clearly documented lung injury associated with cyclical recruitment, the authors did not study the comparative effects of stretch.

The progression of pulmonary injury in the nondependent regions of moderate to severe stretch in our model (tidal volume of 28 ml/kg) is consistent with previous studies investigating isolated stretch injury in intact rabbits (2,24,43). Comparable tidal volumes in normal rabbits (25 ml/kg) ventilated with 50% oxygen for 4 h led to moderate lung injury with modest but significant increases in injury scores, BAL neutrophil counts, and chemokine (MCP-1, GRO) expression (43). Less severe stretch (tidal volume of 15–21 ml/kg) for 4–8 h resulted in increased chemokines (2,24) and/or neutrophils (24) but not edema. Similar to our findings, TNF has not been identified in rabbit models of acute lung injury in the absence of lipopolysaccharide (2,32). Direct comparison of our results for nondependent lung regions with other animal models of stretch is difficult because the effects of tidal volume and time of ventilation appear to be dependent on animal size. Larger animal models (e.g., pigs, dogs, sheep) generally require larger tidal volumes and longer durations of ventilation to induce injury (6,12,15). On the other hand, rodents are much more susceptible to the damaging effects of moderately high tidal volumes, even with very short durations of ventilation (12,18,39,50, 52).

Limitations

We did not have the technical capability to specifically align sections of lung tissue, sampled for histopathology and chemokine measurement, with known regions of cyclical recruitment, atelectasis, or stretch. Although CT (16,28,42) and dCT (10,31,35) can be used to delineate the boundaries of these regions in the intact lung in vivo, the remaining, and substantial, technical challenge would be to define the borders of these regions in vivo with markers that could then be used to guide sampling in the excised lung. Lacking this ability, we chose to analyze the entire lung in sequential slices oriented parallel to the gravitational plane. Because the borders between overdistended, normally ventilated, cyclically recruited, and atelectatic lung regions do not necessarily align with the gravitational plane (28), the expected effect would be that our sampling averaged out some of the spatially specific information and thereby lost statistical power. We would anticipate, however, that the direction of changes in lung injury markers between cyclically recruited lung and overstretched lung would be preserved by sequential slices that include the entire lung. For some of the injury markers, the statistical power of our study was clearly adequate to detect dependent to nondependent changes.

Ventilator settings in our study were adjusted according to the goals for the shunt fraction oscillations (5). The resulting tidal volumes ranged from 24 to 35 ml/kg, a range that has been considered by other investigators to result in moderate stretch (2,24,43). Because the injurious effects of overdistention are known to be quite nonlinear with tidal volume (12), it is likely that a range of tidal volumes only slightly larger than those in our study would have created more injury in the nondependent regions and altered the relative amount of damage between dependent and nondependent regions. For the same reason, however, less stretch would likely have resulted in even less injury in nondependent regions (2). Clinically relevant tidal volumes are well below the range we studied, even when accounting for the “baby lung” effect (17).

The most obvious implication of our study for clinical ventilator management is that priority should be given to avoiding cyclical recruitment. Several aspects, however, limit direct translation to clinical practice. First, we studied a surfactant depletion model of lung injury, a model that is known to be highly recruitable (26,49) and may well be more prone to cyclical recruitment than other models of lung injury (5,45). Second, our study was in a model of lung injury in adult rabbits, and the importance of species differences, lung size, lung maturity, and injury model in determining the dynamics of recruitment and collapse are not currently known. How frequently or infrequently cyclical recruitment occurs in human acute respiratory distress syndrome is also currently unknown. Third, our study examined relatively short time periods. Finally, although the general ranges of ventilator settings that might result in cyclical recruitment are known (namely large plateau pressures, slow rates, and low PEEP) (5), our study also showed that, in individual cases, the specific settings (rate, plateau pressure, PEEP, inspiration to expiration ratio) that produced cyclical recruitment varied between individuals (supplemental data available online at the *Journal of Applied Physiology* website), even under these carefully controlled experimental conditions. Adjusting ventilator settings in clinical practice to avoid cyclical recruitment, therefore, may well require a means to assess cyclical recruitment in patients. Although several methods have shown promise for clinical use, including electrical impedance tomography (20) and dynamic CT (10,31,35), no method capable of documenting cyclical recruitment is currently in routine clinical use.

In summary, previously, an inability to directly measure cyclical recruitment has limited the ability to define its contribution to VALI. Our study used a recently developed tool to study cyclical recruitment and its effects on inflammation. In this pure surfactant depletion model, cyclical recruitment was more damaging than stretch, but whether this is true in other models or patients with acute respiratory distress syndrome remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Terry Freeman for analysis software, Jamie Hayden for microscopy support, Adrienne French for reviewing the histopathological scoring system, Alberta Belli, Mark Campbell, Melissa Java, and Jennifer A Magilton for technical support, and Gabriela Seiler for acquiring the spiral CT images (supplemental data Fig. S3).

GRANTS

This study was supported by National Institutes of Health Grants GM-59274, HL-59052, and GM-64486; Deutsche Forschungsgemeinschaft MA2398/2, and PF424/1; and University of Pennsylvania M. J. Ryan Veterinary Hospital Emergency Service.

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Fig. 1. Lung chemokines. Monocyte chemoattractant protein-1 (MCP-1; *A*), interleukin-8 (IL-8; *B*), and growth-related oncogene (GRO; *C*) production in lung tissue supernatant from regional lung sections as detected by ELISA. All results were normalized to the weight of solids in the individual lung slice. *Lung slice 1* is the most nondependent, and *lung slice 9* is the most dependent. Control rabbits (●) were killed immediately after vascular access was established. The 0-h rabbits (○) were killed immediately after lavage. The remaining rabbits were ventilated for 1.5 h (■), 3 h (□), or 6 h (◆) with high tidal volume, low respiratory rate, and low positive end-expiratory pressure to produce nondependent stretch and dependent cycling atelectasis.

The error bars are 2 times the square root of residual mean squares from a standard variance table and represent the residual variability in the population after accounting for variations due to time and slice number.

Fig. 2.

Lung tissue enzyme activity. Inducible nitric oxide synthase (iNOS) activity in lung tissue supernatant (A) and myeloperoxidase (MPO) activity in lung tissue homogenates (B) from regional lung sections as detected by radioactive citrulline assay and colorimetric assay, respectively. *Lung slice 1* is the most nondependent, and *lung slice 9* is the most dependent. ● Control rabbits; ○, rabbits ventilated after lavage for 0 h; ■, rabbits ventilated after lavage for 1.5 h; □, rabbits ventilated after lavage for 3 h; ◆, rabbits ventilated after lavage for 6 h. The error bars are 2 times the square root of residual mean squares from a standard variance table. OD, optical density; prot, protein.

Fig. 3.

Wet-to-dry equivalent. Wet-to-dry equivalent from regional lung slices based on the ratio of the original lung slice weight to the weight of the sample solids. *Lung slice 1* is the most nondependent, and *lung slice 9* is the most dependent. ● Control rabbits; ○, rabbits ventilated after lavage for 0 h; ■, rabbits ventilated after lavage for 1.5 h; □, rabbits ventilated after lavage for 3 h; ◆, rabbits ventilated after lavage for 6 h. The error bar is 2 times the square root of residual mean squares from a standard variance table.

Fig. 4.

Regional neutrophils. Average intra-alveolar neutrophils counted from 10 hematoxylin and eosin-stained $150 \times 200 \mu\text{m}$ histopathological sections from each regional lung slice. *Lung slice 1* is the most nondependent, and *lung slice 9* is the most dependent. ● Control rabbits; ○, rabbits ventilated after lavage for 0 h; ■, rabbits ventilated after lavage for 1.5 h; □, rabbits ventilated after lavage for 3 h; ◆, rabbits ventilated after lavage for 6 h. The error bar is 2 times the square root of residual mean squares from a standard variance table.

Fig. 5. Photomicrographs of regional lung slices. Images ($150 \times 200 \mu\text{m}$) of hematoxylin and eosin-stained lung slices from the most nondependent regions and dependent regions, respectively, of control rabbits, and those ventilated for 0 h and 6 h after lavage.

Table 1

Statistical results

	Time	Lung Ht	Interaction	Time ²
IL-8	<0.001	<0.001	NS	<0.001
MCP-1	<0.001	0.033	NS	<0.001
GRO	<0.001	NS	NS	0.008
iNOS	0.003	NS	NS	NS
MPO*	NS	NS		
W/De	NS	0.004	<0.001	NS
I-A PMN	<0.001	<0.001	NS	<0.001

Lung Ht, fractional height of the slice; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; iNOS, inducible nitric oxide synthase; GRO, growth related oncogene; MPO, myeloperoxidase; W/De, wet-to-dry equivalent; I-A PMN, intra-alveolar neutrophils; NS, nonsignificant.

* Nonparametric analysis.